DOI 10.1099/mic.0.28041-0

Calcium influences cellular and extracellular product formation during biofilm-associated growth of a marine *Pseudoalteromonas* sp.

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Received 16 March 2005
Revised 6 June 2005
Accepted 7 June 2005

Bacteria undergo a variety of physiological changes following a switch from planktonic growth to surface-associated biofilm growth. Here, it is shown that biofilm development of a marine isolate, *Pseudoalteromonas* sp. 1398, results in global changes in its cytosolic and extracellular proteomes. Calcium influences these proteome responses, and affects the amount of surface-associated biomass and extracellular matrix material produced by *Pseudoalteromonas* sp. 1398. Four extracellular proteins, characterized by N-terminal sequencing, showed increased abundances, while one protein, flagellin, showed reduced abundance at higher [Ca^{2+}]. Immunoblotting and transmission-electron-microscopy analysis confirmed that higher [Ca^{2+}] and surface-associated growth results in the repression of flagella production. Two-dimensional gel electrophoresis (2DGE) studies combined with cluster analysis of global proteome responses demonstrated that Ca^{2+} had a greater regulatory influence on *Pseudoalteromonas* sp. growing in biofilms than on planktonic cultures. Approximately 22 % of the total cytosolic proteins resolved by 2DGE had differing abundances in response to a switch from planktonic growth to surface-associated growth when the cells were cultivated in 1 mM Ca^{2+}. At higher [Ca^{2+}] this number increased to 38 %. Fifteen cellular proteins that were differentially expressed in response to biofilm growth and/or Ca^{2+} were analysed by N-terminal sequencing and/or MS/MS. These proteins were identified as factors involved in cellular metabolic functions, putative proteases and transport proteins, although there were several proteins that had not been previously characterized. These results indicate that Ca^{2+} causes global changes in matrix material, as well as in cellular and extracellular protein profiles of *Pseudoalteromonas* sp. 1398. These changes are more pronounced when the bacterium grows in biofilms than when it grows in planktonic culture.

INTRODUCTION

Many bacteria are successful at adapting to a variety of environmental conditions, in part because they are effective at sensing environmental stimuli and optimizing gene expression patterns. This has been demonstrated for bacteria growing in biofilms, micro-organisms associated with surfaces often encased in extracellular matrix material. During biofilm development, bacteria demonstrate physiological and sometimes morphological changes, which ultimately lead to the formation of a mature biofilm (Dalton *et al.*, 1994; Davies *et al.*, 1993; Sauer *et al.*, 2002; Whiteley *et al.*, 2001). Proteomic studies have demonstrated differences in the amounts of as many as 50 % of the cellular proteins during a switch from planktonic growth to biofilm formation and development (Sauer *et al.*, 2002). These physiological changes suggest that bacterial regulatory elements sense the physical and/or chemical signals associated with surfaces, and respond to these environmental stimuli through gene induction and gene repression events.

In marine environments, calcium is an element that is often

Abbreviations: 2DGE, two-dimensional gel electrophoresis; MS/MS, tandem mass spectrometry; SCLM, scanning confocal laser microscopy; TEM, transmission electron microscopy.

A figure showing 2DGE of protein extracts is available as supplementary data with the online version of this paper.

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enriched on surfaces, either as sedimentary calcium deposits or in association with other marine organisms. Therefore, calcium may be one of the factors that bacteria sense during biofilm-associated growth. Calcium is known to be important for bacterial biofilm formation (see Geesey *et al.*, 2000 for a review). In particular, calcium is involved in specific and non-specific interactions between cells and the substrata (Craven & Williams, 1998; Kallstrom *et al.*, 2000; Matsumoto *et al.*, 2000; Waligora *et al.*, 1999). Calcium-binding proteins are often involved in bacterial adhesion to a surface, and can be important for cell–cell aggregation (Espinosa-Urgel *et al.*, 2000; Hinsa *et al.*, 2003; Rose, 2000). Calcium is also important as an ionic cross-bridging molecule for negatively charged bacterial polysaccharides. Kierek & Watnick (2003) demonstrated that Ca^{2+} plays a role in the architecture of *Vibrio cholerae* biofilms. In particular, when Ca^{2+} was removed from the medium, as may occur when cells are first exposed to a marine environment then switched to a freshwater system, the biofilms disintegrate (Kierek & Watnick, 2003). The results demonstrated that Ca^{2+} is important for the ionic cross-bridging of the O-antigen polysaccharide in the biofilm matrix of *V. cholerae.*

This role of Ca^{2+} has been established (Geesey *et al.*, 2000; Kierek & Watnick, 2003); however, Ca^{2+} may also play a signalling role in bacterial gene expression, particularly during biofilm-associated growth where calcium concentrations may be elevated. Calcium is an important regulatory molecule in eukaryotic cells, but less is known about its role as a regulator in prokaryotes. There is evidence for calcium-mediated regulation in bacteria, including calcium regulation of channels and transporters (Holland *et al.*, 1999; Michiels *et al.*, 2002; Norris *et al.*, 1996). In addition, calmodulin-like proteins are widespread in prokaryotes (Yang, 2001). In *Pseudomonas aeruginosa*, Ca^{2+} acts as a regulatory molecule, and influences the production of extracellular enzymes and toxins, including extracellular proteases that increase at high [Ca^{2+}], and toxins secreted by the Type III secretion system that are repressed by high [Ca^{2+}]. In addition, the amount of extracellular polysaccharide material of an alginate-producing strain of *Pseudomonas aeruginosa* is induced as much as eightfold by Ca^{2+} (Sarkisova *et al.*, 2005). Therefore, Ca^{2+} may have dual roles in biofilm formation, as both an ionic cross-bridging molecule for matrix materials and a sensory ion for gene expression of biofilm-associated components.

In this study, we tested the effect of Ca^{2+} on a marine *Pseudoalteromonas* sp. isolate during biofilm-associated growth. *Pseudoalteromonas* spp. are bacteria found in high numbers in a variety of marine environments (Skovhus *et al.*, 2004). In addition to free-swimming cells, *Pseudoalteromonas* spp. are often found attached to surfaces, including inanimate objects and other marine organisms (Egan *et al.*, 2000; Holmstrom & Kjelleberg, 1999). Since Pseudoalteromonads successfully inhabit many marine environments, including biofilms, it is likely that they respond to multiple signals during changes in environmental conditions, such as those that would occur during biofilm formation and development.

Several experimental approaches have been used to characterize the regulatory changes that occur in bacteria during biofilm development. These approaches have included: microarray analyses (Ren *et al.*, 2004; Schoolnik *et al.*, 2001; Whiteley *et al.*, 2001; Zhu & Mekalanos, 2003), *in vivo* expression technology (Finelli *et al.*, 2003), mRNA subtractive hybridization (Sauer & Camper, 2001), transposon mutagenesis studies (O'Toole & Kolter, 1998) and proteomic studies (Sauer & Camper, 2001; Sauer *et al.*, 2002). Advances in two-dimensional gel electrophoresis (2DGE) allow high resolution of protein spots, and cluster analysis of the protein profiles provides information on global changes of protein amounts following changes in environmental conditions. Proteomic technologies are particularly useful for analysis of uncharacterized bacteria, since these technologies do not require a genetic system for manipulating the bacterial DNA. In addition, although useful, a complete genome sequence is not necessary for proteomic studies. Therefore, proteomic approaches can be useful for studying recent environmental isolates, such as the marine bacterium characterized here. The goal of this study was to characterize the physiological changes that occur in a marine isolate, *Pseudoalteromonas* sp. 1398, during biofilm formation, and to determine if the bacteria respond to calcium during a switch from planktonic growth to biofilm formation.

METHODS

Organism and growth conditions. The bacterial isolate *Pseudoalteromonas* sp.1398 used in this study was originally isolated from marine sediments, and was provided by Dr Keith Cooksey (Montana State University; Wigglesworth-Cooksey & Cooksey, 2005). The bacterium was isolated by washing sediments extensively to remove loosely adhered bacteria, and then sonicating to release the tightly adhered cells. The dislodged bacteria were serially diluted, plated on 2216 marine medium (Difco), and individual colonies isolated. The isolate used in these studies was identified as a *Pseudoalteromonas* sp. by sequence analysis of its 16s rDNA.

Minimal marine medium (MMM) used in these studies was composed of the following (per litre): 18 g NaCl, 5 g $MgSO_4$, 1 g NH_4Cl, 0·6 g KCl, 1 g glucose, 5 mg K_2HPO_4, 0·05 g $NaNO_3$, 0·15 g $NaSiO3.9H_2O$, 8 ml Tris buffer (1·45 M Tris/HCl, 0·55 M Tris Base, pH 7·6), 10 µl trace metals solution, 1 ml vitamins solution. Trace metals solution contained (per litre): 380 mg $FeCl_3.6H_2O$, 430 mg $MnCl_2.4H_2O$, 2 mg $CoCl_2.6H_2O$, 66 mg $ZnSO_4.7H_2O$, 340 mg H_3BO_3, 0·37 mg $CuSO_4.5H_2O$, 3 g Na_2EDTA. Vitamins solution contained (per litre): 0·5 g thiamine, 1 mg biotin, 0·2 mg vitamin B_{12}. Stock cultures were maintained on rich medium, which contained (per litre): 16·7 g NaCl, 5 g $MgSO_4$, 1 g NH_4Cl, 0·6 g KCl, 1 g glucose, 5 g yeast extract, 10 g tryptone. Basal salts medium was composed of the following (per litre): 18 g NaCl, 5 g $MgSO_4$, 0·6 g KCl. $CaCl_2$ was added to the media to produce final concentrations of 0·25, 1·0, 5·0 and 10 mM.

Growth analysis of planktonic and biofilm cultures. For analysis of planktonic cells, bacteria were grown in MMM in culture

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flasks (1 : 100 inoculum) with shaking (250 r.p.m.) at room temperature. Measurements of turbidity (OD_{500}) were taken every 2 h, and the amount of total cellular protein was determined every 6 h.

Biofilm growth experiments were performed with biofilms cultivated in flowing systems on hydrophobic or hydrophilic surfaces that included Teflon mesh, glass coverslips and silicon tubing. Biofilm growth on Teflon mesh was performed in once flow-through 1 litre bioreactors. Planktonic cultures (700 ml of an 18 h culture) were used to inoculate the reactor containing the mesh. Cells were allowed to attach for 60 min, and then the cell suspension was replaced with fresh minimal medium. Flow of fresh medium was initiated at 2 ml min^{-1}. For biomass analysis, the mesh was removed from the reactor, and washed twice in marine salts buffer (MMM without glucose) to remove planktonic and loosely adhered cells. Attached cells were then removed by ultrasonication with a probe-type cell-disruptor (Cole Parmer Instruments) for 3 min, with intermittent ultrasonication cycles of 10 s on and 10 s off. The amount of total cellular protein for attached bacteria was determined per unit surface area by using the bicinchoninic acid (BCA) assay (Pierce Biotechnology). Biofilm growth on glass coverslips was determined by using the once flow-through system as described previously (Nivens *et al.*, 2001). The flow-cell system consisted of a medium reservoir, pump, silicon tubing, a flow-cell and a waste container. The flow-cell contained a glass coverslip (5·3 cm^2) as the substratum, which was sealed to a polycarbonate support with a Viton gasket. Biofilms were removed from the surface and analysed as described for the Teflon mesh experiments. Biofilms were also cultivated on silicon tubing (size 18 Masterflex tubing 30–60 cm) as described by Sauer & Camper (2001). The silicone tubing was filled with marine salts buffer, and the biofilms extracted with the plunger of a 5 ml syringe. Biofilms in each reactor type were cultivated for 7 to 106 h at room temperature.

Microscopic analysis of biofilms. Scanning confocal laser microscopy (SCLM) and light microscopy experiments were performed on biofilms cultivated on glass coverslips using the once flow-through system (Nivens *et al.*, 2001). Stacks of confocal images (512 × 512 bit resolution) were collected using a Leica TCS NT SCLM equipped with a ×100 objective. Bacteria were observed following staining of the cells for 20 min with LIVE/DEAD *Bac*Light stain (Molecular Probes). Three-dimensional reconstructions of the images were made using a maximum projection of the image stacks. The total surface-associated biomass was observed with reflected laser light of combined 488 and 586 nm wavelengths. Biofilms were stained with alcian blue (0·1 % in water), and the extracellular acidic polysaccharide surrounding the bacteria (Gerhardt, 1994) observed with a Olympus PH2-RFCA light microscope with ×100 objective. Transmission electron microscopy (TEM) was performed on 18 h planktonic cultures and on biofilms cultivated on silicone tubing (106 h biofilms). For TEM, bacteria were fixed with 25 % glutaraldehyde, applied to 300 mesh Formvar coated cooper grids, stained with 1 % uranyl acetate and analysed with a Zeiss 100CA transmission electron microscope.

Extracellular protein analysis. Extracellular proteins were characterized from 18 h planktonic cultures or from biofilms (106 h on silicone tubing). Cells were harvested from the walls of the tubing as described above, and removed from the medium by centrifugation (twice for 15 min at 10 000 ***g***). Proteins from the supernatants were precipitated by adding ice-cold trichloroacetic acid (15 % final concentration) followed by incubation on ice for 1 h. TCA-precipitated extracellular proteins were pelleted by centrifugation, and the pellets were washed twice with ice-cold acetone. Protein pellets were resuspended in solubilization buffer (8 M urea, 2 M thiourea, 35 mM DTT, 2 % SDS, 1 mM EDTA, 10 mM Tris/HCl pH 8·0). The protein concentrations were determined by a modified BCA method (Pierce). Protein samples (5 μg) were resolved by 12 % SDS-PAGE (Laemmli, 1970). Proteins were stained with either silver nitrate or Coomassie blue. Immunoblotting was used for flagellin determinations. Proteins were electroblotted onto nitrocellulose membranes and probed with antibodies against the flagellin of *Pseudomonas aeruginosa* strains PAK and PAO1 (generously provided by Dr Daniel Wozniak, Wake Forest University, Winston-Salem, NC, USA). Horseradish-peroxidase-conjugated anti-rabbit IgG was used as the secondary antibody, and flagellin bands were detected using chemiluminescence (Ausubel *et al.*, 1988).

2DGE. Following removal of cells from the Teflon surface as described above, cells were centrifuged and the cell pellets were resuspended in 200–400 μl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8·0), containing 0·3 mg PMSF ml^{-1}. The cells were disrupted by ultrasonication for 2 min with 10 s on and 10 s off cycles. Unbroken cells and debris were removed by centrifugation at 15 000 r.p.m. for 30 min, and cell-free crude protein extracts were either stored at −20 °C or used immediately for further studies. Protein concentrations were determined by BCA assay (Pierce).

Protein 2DGE was performed according to published procedures (Gorg *et al.*, 1995, 2000; Gorg, 1999; Rabilloud, 2000) as modified by Sauer *et al.* (2002). The first dimension of protein separation was carried out using Immobiline IPG (immobilized pH gradient) DryStrips (18 cm, pH 4–7, linear; Amersham). The IPG strips were rehydrated with the protein sample (100–700 μg total protein) in 450 μl rehydration solution (11 M urea, 2·7 M thiourea, 35 mM DTT, 3 % CHAPS, pharmalyte pH 3–10). The strips were incubated overnight at room temperature under mineral oil. After rehydration, IEF was carried out for a total of 35 kVh at 20 °C, under mineral oil, using a Multiphor system (Amersham Biosciences). Following this, the IPG strips were equilibrated in DTT and iodoacetamide equilibration buffers (6 M urea, 0·5 M Tris/HCl, 30 % glycerol, 4 % SDS, pH 6·8), and layered onto 10 % SDS-PAGE gels electrophoresed using a DALT12 system (Amersham Biosciences). Molecular mass markers (Bio-Rad) were run in the same gel, or in a parallel gel under identical conditions. Protein spots were detected by staining with silver nitrate or colloidal Coomassie blue G250.

Cluster analysis of protein spots. Differential analysis of 2DGE protein maps was performed with the Progenesis (Nonlinear Dynamics) software package. This software detects protein spots, subtracts the background and assigns signal intensity value to each spot. Signal intensities were normalized against the total signal intensity of all the spots on a gel multiplied by 100. The corresponding spots were matched between the gels using the Progenesis combined warping and matching algorithm. Spots with a normalized volume above 0·02 and averaged over two gels (from independent growth experiments) were recorded. Cluster analysis was performed by using data files generated by Progenesis and exported to Microsoft Excel. Plots of the $\log_{10}$ values for each spot signal intensity were performed using GeneSpring software (Silicon Genetics).

Protein identification. N-terminal sequencing and tandem mass spectrometry (MS/MS) were used for the identification of proteins. For N-terminal sequencing, the gels were equilibrated for 20 min in blot buffer (according to the manufacturer's guidelines; Hoefer Dalton) and the proteins electroblotted onto Immobilon-P PVDF membranes (Millipore). Electroblotting was carried out for 2 h at 300 mA constant current in a Hoefer Dalton Trans-Blot unit. The PVDF membranes were stained with 0·1 % Coomassie blue G250 in 50 % methanol/7 % acetic acid for 5 min, and destained with 50 % methanol. The membranes were washed with water and allowed to dry before storage at −20 °C. The protein spots of interest were excised from the membranes, and N-terminal amino acid sequenced by Midwest Analytical (http://www.mastl.com), using the Edman degradation method. Protein identification was performed using a protein BLAST search for short nearly exact sequences at

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http://www.ncbi.nlm.nih.gov/blast (Altschul *et al.*, 1997). For MS/MS analysis, the spots of interest were excised from Coomassie blue stained gels and trypsin digested. MS/MS analysis was performed by Proteomics Centre, Genome BC, University of Victoria (www.proteincentre.com), using a Sciex linear ion trap quadrupole LC-MS/MS mass spectrometer (Applied Biosystems). Protein identification of MS/MS data was performed by using BLAST at http://dove.embl-heidelberg.de/Blast2/msblast.html (Shevchenko *et al.*, 2001).

RESULTS

Ca^{2+} does not affect the growth rate of *Pseudoalteromonas* sp. in planktonic culture, but affects biofilm-associated growth on both hydrophobic and hydrophilic surfaces

Since calcium influences biofilm formation for many bacteria, we first determined if this effect extended to the *Pseudoalteromonas* sp. isolate used here. For these studies, the bacteria were allowed to attach to and grow on Teflon mesh or glass surfaces in the presence of differing [Ca^{2+}] (0·25, 1·0, 5·0 and 10 mM as $CaCl_2$). Cells were then removed from the surfaces and assayed for total protein as a measure of the surface-associated biomass. In planktonic culture, the [Ca^{2+}] in the media had little effect (less than 15 %) on the growth rate of *Pseudoalteromonas* sp. 1398; the growth curves for each of the culture conditions were essentially identical (Fig. 1A). However, the addition of $CaCl_2$ had a significant effect on the biofilm-associated growth of *Pseudoalteromonas* sp. 1398 on both hydrophobic and hydrophilic surfaces (Fig. 1B,C). On glass surfaces, 10 mM $CaCl_2$ resulted in a 20-fold to 100-fold greater amount of surface-associated biomass compared to biofilm growth in medium with 0·25 mM $CaCl_2$. Less total biomass was observed on the Teflon surface compared to the glass surface (Fig. 1B). However, [Ca^{2+}] affected the total amount of biomass on this hydrophobic surface as well. Therefore, although [Ca^{2+}] does not appear to influence *Pseudoalteromonas* sp. 1398 growth rate in planktonic culture, it significantly impacts its ability to colonize both hydrophobic and hydrophilic surfaces.

Scanning confocal laser microscopy (SCLM) was used as a second method to determine the effect of Ca^{2+} on *Pseudoalteromonas* sp. biofilm formation. Fig. 2 (A–D) shows SCLM images of a *Pseudoalteromonas* sp. biofilm after 30 h growth when the [Ca^{2+}] was varied from 0·25 to 10 mM. The *Bac*Light staining of the biofilms did not show significant amounts of nonviable cells associated with biofilms. At low [Ca^{2+}] the surface contained single cells and small groups of cells with few bacteria extending more than one cell deep from the surface. In contrast, the presence of 1 and 5 mM $CaCl_2$ resulted in the formation of cell clusters that extended 20 to 40 μm from the surface. Thicker biofilms (approximately 50 μm) formed in the presence of 10 mM $CaCl_2$. These experiments demonstrated that the increased biomass associated with the surface observed with the total protein analyses was due to the formation of thicker microcolonies that extend from the solid substratum.

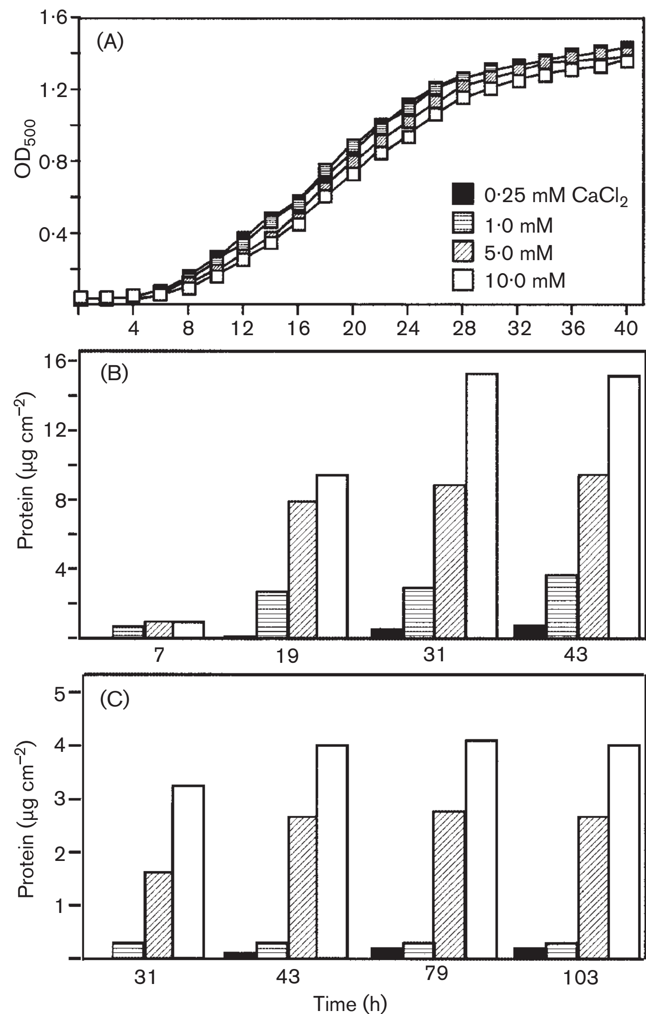

Fig. 1. Growth curves of *Pseudoalteromonas* sp. 1398 in minimal medium at differing [$CaCl_2$]. (A) Growth in planktonic culture for cells in 0·25, 1·0, 5·0 and 10·0 mM $CaCl_2$. Total cellular protein of *Pseudoalteromonas* sp. 1398 biofilms associated with (B) glass surfaces and (C) Teflon surfaces. Biofilms were cultured in MMM with 0·25, 1·0, 5·0 and 10·0 mM added $CaCl_2$.

Ca^{2+} influences the amount of extracellular matrix material associated with the biofilm

In a mucoid strain of *Pseudomonas aeruginosa*, Ca^{2+} caused increased production of the exopolysaccharide alginate during biofilm formation (Sarkisova *et al.*, 2005). Since Ca^{2+} also affects the biomass and thickness of the biofilms of this marine *Pseudoalteromonas* sp., it is possible that this increased thickness is due to an increased amount of extracellular polysaccharide matrix material produced by the bacteria. To test this hypothesis, we performed alcian blue staining of the biofilms at various [Ca^{2+}]. The results in Fig. 2 (E–H) show increased amounts of stained material surrounding the cell clusters with increased [Ca^{2+}]. Since alcian blue stains acidic polysaccharides, the results

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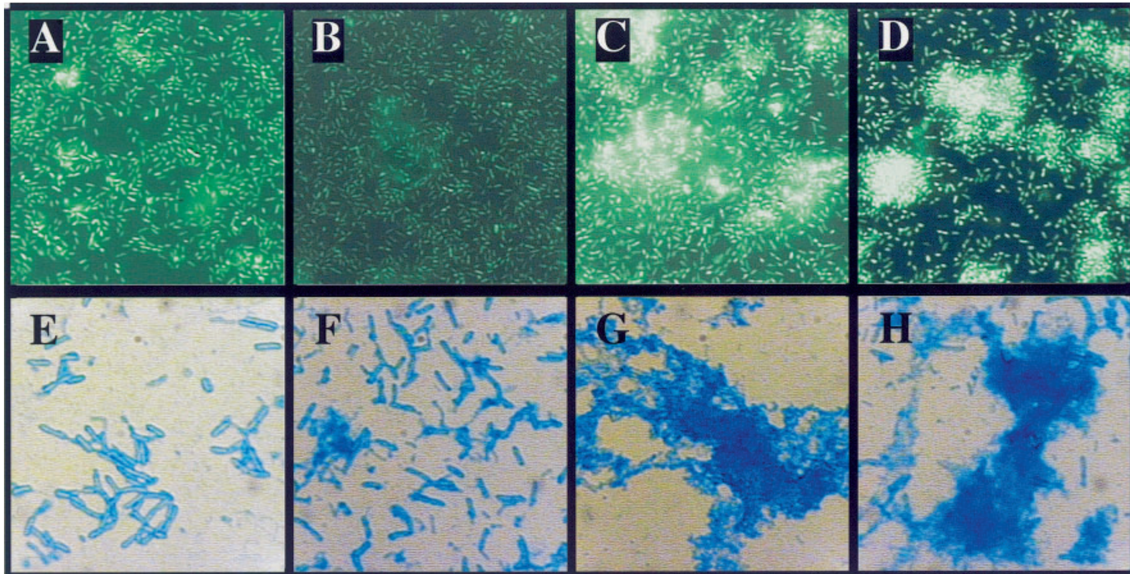

Fig. 2. SCLM images of *Pseudoalteromonas* sp. 1398 biofilms, cultivated for 30 h on glass surfaces at different [$CaCl_2$]: (A) 0·25 mM, (B) 1·0 mM, (C) 5·0 mM and (D) 10·0 mM $CaCl_2$. Alcian blue stained *Pseudoalteromonas* sp. 1398 biofilms cultivated at different [$CaCl_2$]: (E) 0·25 mM, (F) 1·0 mM, (G) 5·0 mM and (H) 10·0 mM $CaCl_2$.

indicate that this *Pseudoalteromonas* sp. produces larger amounts of biofilm-associated polysaccharide with increased [Ca^{2+}].

Similar results for the amount of exopolysaccharide were observed with TEM of the bacteria (Fig. 3). In planktonic culture, the cells cultured at low [Ca^{2+}] had single polar flagella, and did not appear to synthesize detectable levels of extracellular matrix material (Fig. 3A). At 10 mM $CaCl_2$, the planktonic cells appeared to lose their flagella, and instead produced material that was probably polysaccharide (Fig. 3B). When grown in biofilm at low [Ca^{2+}], the bacteria produced small amounts of extracellular material (Fig. 3C). The amount of this matrix material increased at higher concentrations of calcium (Fig. 3D), confirming the alcian blue staining results. No flagella were associated with the cells cultivated in biofilms (although some broken flagella were observed as debris in these cultures).

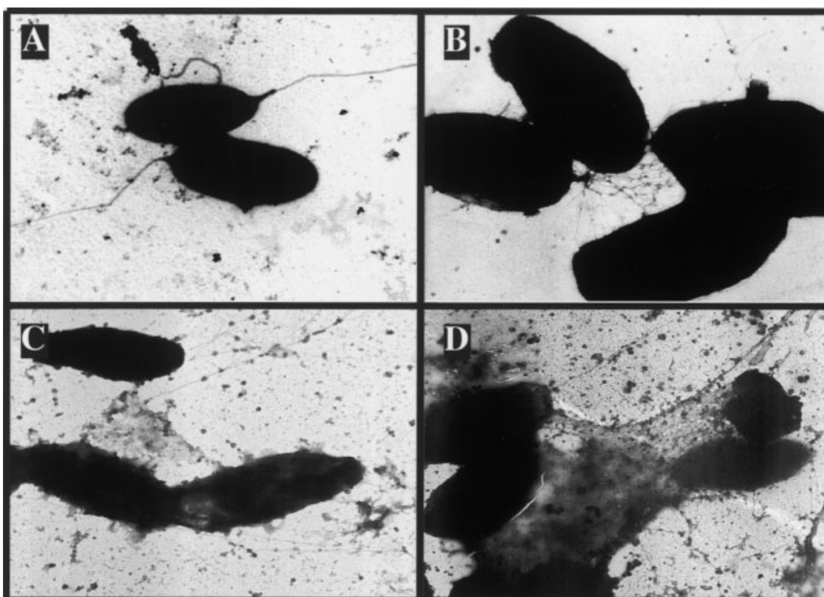

Fig. 3. TEM images of *Pseudoalteromonas* sp. 1398. (A) Cells grown in planktonic culture with 0·25 mM $CaCl_2$, showing polar flagella. (B) Cells grown in planktonic culture with 10·0 mM $CaCl_2$, showing production of intercellular matrix material but no flagella. (C) Cells from a biofilm cultivated in 0·25 mM $CaCl_2$, showing some matrix material, but no flagella. (D) Cells from a biofilm cultivated with 10·0 mM $CaCl_2$, showing an increased amount of matrix material.

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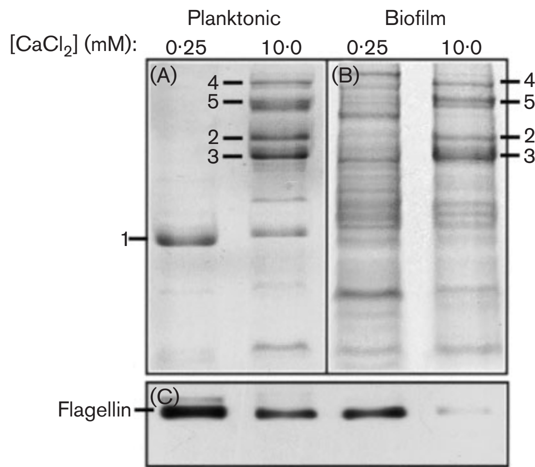

Fig. 4. Extracellular proteins produced by *Pseudoalteromonas* sp. 1398 during growth in MMM supplemented with 0·25 or 10·0 mM $CaCl_2$. (A) Growth in planktonic culture. Proteins were resolved by SDS-PAGE and stained with Coomassie blue. (B) Growth in biofilm culture. The results of N-terminal and MS/MS sequence analysis of proteins 1–5 are shown in Table 1. (C) Immunoblot analysis of cells cultivated using the same conditions as for the Coomassie blue-stained gels, with antibodies raised against flagellin from *Pseudomonas aeruginosa*.

Table 1. N-terminal and MS/MS analysis of extracellular proteins produced by *Pseudoalteromonas* sp. 1398

Protein	Peptide sequence	Protein with highest similarity*
1†	ALYVNTNVSALNAQR	Flagellin (*Aeromonas punctata*) 93 %
2‡	GVDGADGADGVDG	Predicted phosphatase (‘*M. degradans*’) 92 %
3‡	**N-terminal analysis**	
	GVLPDNQMKSDXYSA	Unidentified protein
	MS/MS analysis	
	HYTLGR	
	AAFLESR	
	RNFDNLPR	
	FQGDEQVSR	
	PVLFGGTYR	
	YSSQDDTGGVLFK	
	LNSPVPESEFER	
	ADQPEDLSAGTLYQK	
	SDVLANEVVTK	
4‡	ALLIDEDVEEGEF	Conserved uncharacterized protein
5‡	SSDDDDKIESLTVKNT	Unidentified protein

*Sequence identities are based on the closest homologues identified using BLAST searches as described in Methods. Percentage identity of the peptide to the protein from the organism is indicated.
†Protein up-regulated by low [$CaCl_2$].
‡Protein up-regulated by high [$CaCl_2$].

Ca^{2+} affects extracellular proteins produced by *Pseudoalteromonas* sp. 1398

The TEM results for the planktonic cultures indicated that Ca^{2+} affects the production of flagella by this species of *Pseudoalteromonas*. Since Ca^{2+} influences several secreted factors of *Pseudomonas aeruginosa*, it is possible that it also influences the production of extracellular proteins of *Pseudoalteromonas* sp. To test this, we extracted, TCA precipitated and analysed extracellular proteins from both biofilm and planktonic cultures incubated at differing [Ca^{2+}]. In planktonic cultures at low [Ca^{2+}] one dominant protein band was observed (Fig. 4A, protein 1). This band was blotted onto PVDF membrane, excised and analysed by N-terminal sequence analysis. The N-terminal sequence of this protein had 93 % identity to the flagellin protein, FlaA, of *Aeromonas punctata*. Identification of flagellin under planktonic conditions at low concentration of Ca^{2+} is consistent with the TEM results showing single polar flagella in these cells. To verify that this protein was flagellin, we immunoblotted the TCA-precipitated proteins from biofilm cultures and from planktonic cultures, using an antibody developed against the flagellin of *Pseudomonas aeruginosa* (PAK and PAO1). The results in Fig. 4(C) show a band corresponding to flagellin under all conditions tested: planktonic growth at 0·25 and 10·0 mM Ca^{2+}, and biofilm growth at 0·25 and 10 mM Ca^{2+}. However, in agreement with the TEM results, the greatest intensity of the flagellin band was observed in samples from the planktonic culture at low levels of Ca^{2+}. The least intense band was observed in the sample from the biofilm culture at 10 mM Ca^{2+}. The flagellin observed in the biofilm cultures was likely to be due to the flagellin debris, as was observed by TEM.

When the [Ca^{2+}] was increased to 10 mM, at least four extracellular proteins showed increased amounts in planktonic culture, with the flagellin band showing reduced amounts (Fig. 4A). In biofilm at low [Ca^{2+}], many bands were observed that may have been derived from debris from lysed cells. However, at increased [Ca^{2+}] the four bands that were observed in planktonic culture at high [Ca^{2+}] were also observed in biofilms (Fig. 4B). These proteins were not observed in biofilm culture at lower [Ca^{2+}], indicating that Ca^{2+} has a greater regulatory influence on extracellular protein production in *Pseudoalteromonas* sp. than does biofilm-associated growth. The four extracellular proteins that showed increased expression with Ca^{2+} addition were characterized by either N-terminal sequence analysis or MS/MS sequencing of peptide fragments. The protein labelled 2 (the 71 kDa protein) had a repeating GADGVD sequence, also found in secreted proteins from ‘*Microbulbifer degradans*’, *Corynebacterium glutamicum* and *Bacteroides fragilis*. In ‘*M. degradans*’, the protein showing sequence similarity is predicted to be a secreted phosphatase. The sequence identity for protein 3 (the 64 kDa protein) was too low to make a definitive identification. Protein 4 (the 106 kDa protein) had low levels of sequence similarity to a conserved hypothetical protein from *Clostridium thermocellum*. Protein 5

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(the 85 kDa protein) could not be identified because of its lack of sequence similarity to other known proteins.

Calcium affects the global proteomic response of biofilm-associated *Pseudoalteromonas* sp. 1398, and has less effect on planktonic cells

Since Ca^{2+} affects biofilm formation and extracellular product formation by this bacterium, we also determined if it influences the amounts of cytosolic proteins. For these studies, we first evaluated the proteomic responses of *Pseudoalteromonas* sp. at a baseline $[Ca^{2+}]$ (1·0 mM) by 2DGE. The bacteria were cultivated in planktonic culture for 18 h, and therefore their proteomes contain proteins produced up to and through the exponential phase of growth. Following planktonic growth, the bacteria were allowed to colonize a Teflon surface for 7 h. A comparison of these proteome maps reflects the changes in the physiology of the bacterium when shifted from planktonic growth to the early stage of biofilm development. A 2DGE differential analysis of cytosolic proteins is shown in Fig. 5(A). Differential-comparison analysis of the protein profiles was obtained using Progenesis and GeneSpring software, and Fig. 5(B) shows the distribution of the resultant ratios ($\log_{10}$) for each protein resolved on the 2D gels, for the mean of duplicate samples. The separation lines show the border between the spots with different expression profiles, and the spots along the *x*- and *y*-axis are those found only in the planktonic or sessile biofilm culture, respectively. On average, 800 protein spots were resolved per gel. Of the 800 spots, approximately 200 showed a twofold or greater differential intensities in biofilm versus planktonic cultures under this growth condition (1 mM Ca^{2+}). These include 93 proteins showing increased abundance in biofilms, 47 showing decreased abundance in biofilms and 62 that appear only in the biofilm culture. These differences represent approximately 25 % of the proteins resolved by 2DGE, and demonstrate that *Pseudoalteromonas* sp. cells undergo major physiological changes during a shift from planktonic to biofilm culture.

To determine the role of Ca^{2+} in the proteomic response of *Pseudoalteromonas* sp. 1398 in both planktonic and biofilm culture, we compared protein profiles of cells incubated at 0·25 and 10·0 mM $[Ca^{2+}]$. Fig. 6 shows the ratios of $\log_{10}$ values of the signal intensities for each protein spot for the planktonic (Fig. 6A) and biofilm cells (Fig. 6B). The data

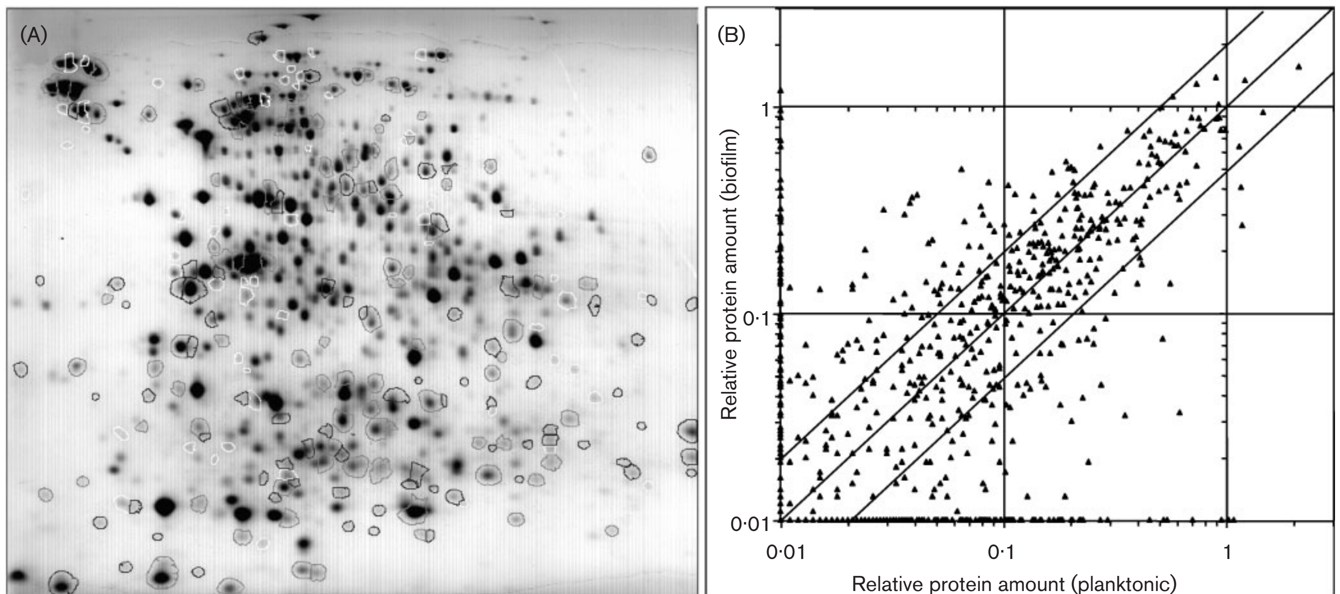

Fig. 5. (A) 2DGE analysis of *Pseudoalteromonas* sp. 1398 cellular proteins. Cells were cultured in a biofilm for 7 h in a once flow-through reactor with minimal medium and 1·0 mM $CaCl_2$. Proteins were resolved using pH 4–7 isoelectric focusing and 10 % SDS-PAGE. Highlighted are protein spots showing different amounts between planktonic cells and surface-associated cells, as determined by Progenesis software for two or more independent experiments. Shown in grey are spots that have twofold or greater increased intensity in biofilm cultures versus planktonic cells. Shown in black are protein spots that were only observed in biofilm cultures, with no corresponding spot in planktonic cultures. Shown in white are spots with twofold or greater increased amounts in planktonic cells versus biofilm cultures. (B) Analysis of signal intensity for each protein resolved by 2DGE. Spot signal intensity from biofilm culture (*y*-axis) plotted against its intensity from planktonic culture (*x*-axis). Each point represents the mean intensity for a protein from two or more gels under each growth condition. Proteins represented above the top line are those with twofold or greater intensity in biofilm culture. Proteins represented below the bottom line are those with twofold or greater intensity in planktonic culture. Proteins represented along the *y*- and *x*-axes are those only observed in biofilm or planktonic culture, respectively.

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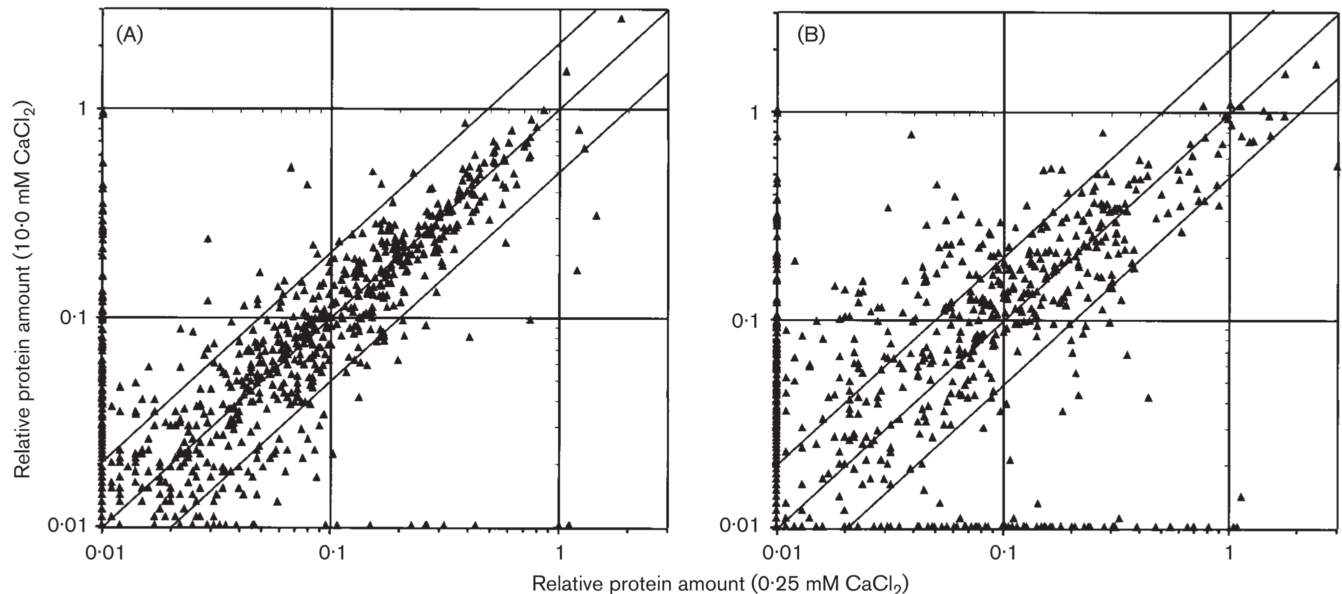

Fig. 6. Cluster analysis of the effect of Ca^{2+} on global protein profiles of planktonic and surface-associated *Pseudoalteromonas* sp. 1398. (A) Individual protein-spot signal intensity for planktonic cultures plotted for cultures incubated in minimal medium with 10 mM $CaCl_2$ (*y*-axis) versus spot intensity in planktonic culture at 0·25 mM $CaCl_2$ (*x*-axis). Spots above the top line and below the bottom line indicate proteins with twofold or greater amounts in 10 and 0·25 mM $CaCl_2$, respectively. Spots along the *y*-axis are proteins only found when cells are cultivated in 10 mM $CaCl_2$, and those along the *x*-axis are those that are only found when cells are grown in 0·25 mM $CaCl_2$ medium. (B) Cluster analysis of individual protein spots for cells cultivated in biofilms, when cells are exposed to 10 (*y*-axis) and 0·25 (*x*-axis) mM $CaCl_2$. All other parameters are the same as in (A). Data represent the mean of intensities from duplicate independent experiments.

distribution had a greater range (a greater scatter of the protein-spot signal intensities) for the biofilm bacteria than for the planktonic cells, indicating that the increased [Ca^{2+}] has less effect on the bacteria grown planktonically, than on biofilm-associated cells. For the planktonic cells, most of the protein spot intensities lie within the twofold range, showing equivalent amounts of protein under both conditions (0·25 and 10 mM $CaCl_2$), with 61 proteins having at least a twofold greater intensity in the 10 mM $CaCl_2$ medium, and 26 proteins only found when the cells were cultured with 10 mM $CaCl_2$ (spots along the *y*-axis). In contrast, the proteomes had a greater number of proteins that were outside of the twofold range for the biofilm cultures cultivated at differing [Ca^{2+}] (Fig. 6B). In this case, 159 proteins had at least a twofold increase in intensity in the 10 mM $CaCl_2$ medium. Of these, 88 proteins were found only under the 10 mM [Ca^{2+}] condition. The results indicate that Ca^{2+} has a regulatory effect (or protein stability effect) on many proteins during planktonic growth. However, [Ca^{2+}] exerts a much more pronounced effect on the proteome of biofilm-associated cells.

Experiments where the bacteria were shifted from planktonic growth to biofilm-associated growth were performed at four [Ca^{2+}] (0·25, 1·0, 5·0 and 10 mM). The results in Table 2 show the cluster-analysis results of proteins with twofold or greater increased or decreased abundance in biofilm culture versus planktonic culture for each [Ca^{2+}]. The results demonstrate that the number of protein spots showing increased intensity in biofilms compared to planktonic cultures increases with increasing [Ca^{2+}]. For example, at 0·25 mM [Ca^{2+}], 174 proteins show changed abundance in biofilm vs planktonic cultures, with 76 showing at least a twofold increase in abundance, 46 appearing only in the biofilm cultures and 52 showing decreased abundance. In contrast, at 10 mM $CaCl_2$ the number of proteins showing increased abundance almost doubled, with 167 showing a twofold or greater increased abundance in biofilm culture, 80 proteins only appearing in biofilm culture, and 55 showing decreased abundance in biofilm culture. These results demonstrate that *Pseudoalteromonas* has a greater regulatory response to a shift from planktonic culture to biofilm culture as the concentration of Ca^{2+} increases.

Cluster analysis and identification of proteins showing differing expression patterns

We grouped the cytosolic proteins into five classes based on the conditions under which they were differentially expressed (for the four different [Ca^{2+}], and with at least two experiments per [Ca^{2+}]). Class I proteins (27 proteins) showed increased abundance in biofilm cells, but were unaffected by Ca^{2+}. An example of a class I protein (spot 1) is shown in the Supplementary Figure (A1–A4, B1–B4

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Table 2. Comparison of the no. of protein differences between surface-associated biofilm cells and planktonic bacteria at differing [Ca^{2+}]

Concn $CaCl_2$ (mM)	Protein differences			
	No. increased in biofilm*	No. newly appeared in biofilm†	No. decreased in biofilm‡	Total
0·25	76	46	52	174
1·0	93	62	47	202
5·0	108	74	45	227
10·0	167	80	55	302

*Proteins present under both biofilm and planktonic conditions, but with at least twofold increased amounts in biofilms for two or more independent experiments.

†Proteins only found in biofilm-cultivated cells and not present in planktonic cultures.

‡Proteins with at least a twofold increased amount in extracts from planktonic cultures compared to extracts of biofilm-cultivated cells.

available with the online journal). N-terminal sequence and/or MS/MS analysis were used to identify peptide sequences for six class I proteins (Table 3). For protein spot 1 both N-terminal sequencing and MS/MS gave identical sequences for the N-terminus of the protein, verifying that both techniques were useful for obtaining peptide sequences. Based on the sequence identity, and consistent with the predicted pI and molecular mass, the protein's most likely homologue is a hypothetical protein from *Pseudomonas syringae*. Protein spot 2 has the highest sequence identity to a hypothetical protein from a marine *Pirellula* sp. (Glockner *et al.*, 2003), and to a putative TonB-dependent receptor from *Sinorhizobium meliloti* (Finan *et al.*, 2001) and *Idiomarina loihiensis* (Hou *et al.*, 2004). Class I proteins spots 9 and 13 could not be identified due to inadequate amino acid sequence identity to known proteins. Protein spots 6 and 8 were identified as the metabolic proteins malate dehydrogenase and succinyl-CoA synthetase.

Class II included 39 proteins that showed increased abundance in biofilms, and were also influenced by Ca^{2+} (Supplementary Figure, C1–C4, D1–D4, shows a representative class II protein- spot 3). These proteins were not found in planktonic cultures at any [Ca^{2+}], but occurred in biofilm cells at low abundance and showed the greatest abundance in biofilms at 10 mM $CaCl_2$. The peptide sequences of six class II proteins are shown in Table 3. Sequence homology searches revealed strong identity for protein 10, identified as PurM. Other proteins also showed identity to known proteins, including protein 5, which has similarity to a TonB-dependent biopolymer transport protein of *I. loihiensis* (Hou *et al.*, 2004), protein 3, which has similarity to the outer-membrane protein OmpW of *I. loihiensis* (Hou *et al.*, 2004), and protein 7, which has similarity to a conserved hypothetical protein. Protein 1270 did not have strong identity to any known proteins.

Twenty-four proteins were grouped as class III, those with decreased intensity in biofilms, but unaffected by Ca^{2+} (example shown in the Supplementary Figure E1–E4 and F1–F4, labelled spots 14 and 15). N-terminal peptide sequences of protein 14, gave strong identity to a protein in peroxiredoxin/glutaredoxin family (Heidelberg *et al.*, 2000). The Supplementary Figure (G1–G4, H1–H4) shows an example of a class IV protein. This class includes 101 proteins that had reduced abundance in biofilms vs planktonic cultures. In biofilm cells the proteins' amounts increased with increasing [Ca^{2+}], but in planktonic cells remained constant. The Class IV protein 12 has strong identity to an intracellular protease of the ThiJ/PfpI family (Glockner *et al.*, 2003). Protein 16 has similarity to a tetratricopeptide repeat (TPR) protein of *I. loihiensis*. Class V includes 70 proteins that are of interest since they do not appear to be regulated by the surface, but are influenced by Ca^{2+}. Sequence information was obtained from one of these proteins, which had a low level of similarity to a hypothetical protein from *Photorhabdus luminescens*.

DISCUSSION

When bacteria adhere to surfaces, they sense chemical or physical properties associated with the surface, and undergo physiological changes. These changes include expression of genes that allow them to firmly adhere to the surface, termed secondary adhesion, and ultimately to develop into structured biofilms. Genes expressed and proteins produced during biofilm formation have been characterized for several organisms (Kachlany *et al.*, 2001; Kuchma & O'Toole, 2000; Perrot *et al.*, 2000; Pratt & Kolter, 1999; Ren *et al.*, 2004; Sauer *et al.*, 2002; Schoolnik *et al.*, 2001; Tremoulet *et al.*, 2002; Whiteley *et al.*, 2001; Zhu & Mekalanos, 2003). Here, we report on the global changes in physiology during the initial stages of biofilm formation of a marine bacterium, *Pseudoalteromonas* sp. 1398. These studies provide information on the physiological response of a marine organism to a shift from planktonic to sessile biofilm growth, and therefore can be used to characterize similarities between biofilm formation in this marine isolate and that in freshwater isolates, as well as bacteria of medical importance. In addition, this study provides information on global changes in protein profiles resulting from a combination of two environmental parameters, surface-associated growth and the concentration of calcium in the medium. Calcium concentration was chosen as an environmental variable since it is known to influence the biofilm formation of certain bacteria (Geesey *et al.*, 2000). Here we show that Ca^{2+} also influences the formation of biofilms by this *Pseudoalteromonas* isolate.

Calcium is thought to allow thicker bacterial biofilms, primarily through ionic bridging of the extracellular matrix material (Korstgens *et al.*, 2001; Rose & Turner, 1998; Rose, 2000). Extracellular matrix material is usually composed of negatively charged extracellular polysaccharides, and the cross-linking of these polysaccharides with divalent cations

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Table 3. Peptide sequences of biofilm and/or calcium-regulated proteins

Class	Protein	Method*	Peptide sequence	kDa†	pI‡	Protein with highest similarity§
I	1	N-terminal	SNETYTFNAELRTATG	23	5·8	Conserved hypothetical protein (*Pseudomonas syringae*), or ribosomal protein (*Pseudomonas putida*, 67 %)
		MS/MS	SNETYTFNAELR			
			LPGAVSSVDLAK			
			VVHFLGEEAVTK			
			VPVHFLDAEAVTK			
			LDFQR			
			LTHLQFDR			
			PAGVSSVDLAK			
			ASLTEFHK			
	2	N-terminal	ETQELLAEYRSVVDE	23	5·2	Putative TonB-dependant receptor (*I. loihiensis*, 67 %)
	6	N-terminal	SVLINKDTKVIXQGF	30	5·5	Succinyl-CoA synthetase (*P. fluorescens*, 100 %)
	8	N-terminal	MKVAVLGAAGGIGQA	31	4·8	Malate dehydrogenase (*I. loihiensis*, 100 %)
		MS/MS	AEGLMTLVPK			
			WLFNVNAGLLK			
			SEAFVAELK			
			FCMSLVK			
			LQNAEGTEVVQGK			
			VFGLTTDVLR			
	9	N-terminal	ATIGGTDVTYGGYIK	34	4·8	No identification
	13	N-terminal	AEQQVQPLHNEKHAN	26	5·8	No identification
II	5	MS/MS	NVDFGQLGR	24	5·2	TonB-system biopolymer transport component (*I. loihiensis*)
			FLDTLEQFLK			
			LTYVAQSFDLK			
			LVADQNASLESFDR			
			AGLQLQTAAVK			
			VDESELLK			
			DAVPFNSDNR			
	3	N-terminal	NLSVNVGAINVNPDN	20	4·2	OmpW (*I. loihiensis*, 60 %)
	10	N-terminal	SEQKQSLSYKDAGVD	34	4·9	PurM (95 %)
	7	N-terminal	AEYDIYGKAEVQIAN	27	5·5	Conserved protein (*Burkholderia fungorum*, 67 %)
	1270	MS/MS	LLGTETFR	52	5·7	No identification
			AEPSQLA			
III	14	N-terminal	MLKNIEGQTIPQVTF	26	5·0	Peroxiredoxin/glutaredoxin family protein (*V. cholerae*, 67 %)
IV	12	N-terminal	AKVLMITGDFVEDYE	34	5·5	ThiJ/PfpI family of proteases (*D. vulgaris*, 86 %)
	16		EEVKTKRVPALREKV	26	5·0	TPR repeats protein (*I. loihiensis*, 67 %)
V	c	N-terminal	GVLPDNQMKSDWYSA	51	4·6	Hypothetical protein *Photorhabdus luminescens*

*Peptide sequences were obtained using either N-terminal or MS/MS analysis as described in Methods.
†Estimated molecular mass of the proteins, based on their positions in the SDS-PAGE gels.
‡Estimated charge of the proteins based on their positions on the IPG strips.
§Sequence identities are based on the closest homologues identified using BLAST searches as described in Methods.

forms a cohesive extracellular gel for biofilms. Calcium-binding proteins may also be important for bacterial adhesion to a surface (Craven & Williams, 1998; Hinsa *et al.*, 2003; Matsumoto *et al.*, 2000; Waligora *et al.*, 1999). In addition to its role in chemical cross-linker, calcium probably influences regulatory processes in bacteria. Therefore, we tested the responses of *Pseudoalteromonas* sp. 1398 to differing [Ca^{2+}] while the cells were undergoing a shift from planktonic growth to surface-associated biofilm growth.

Included in the physiological changes that occur in *Pseudoalteromonas* sp. 1398 in response to both Ca^{2+}, and to a surface, is the production of extracellular matrix material, probably an acidic polysaccharide, which stains with alcian blue. Calcium also influenced extracellular protein production of *Pseudoalteromonas* sp. 1398. Regulation of extracellular proteins by calcium has been demonstrated for other bacteria, including the myxobacterium *Stigmatella aurantiaca* (Chang & White, 1992), where the addition of Ca^{2+} to the growth medium resulted in the formation of extracellular fibrils and the appearance of a 30 kDa fibril protein, and *Pseudomonas aeruginosa*, where the amounts of certain extracellular proteases and toxins are affected by Ca^{2+}. Our results show that the presence of 10 mM Ca^{2+}

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causes changes in extracellular protein biosynthesis and/or accumulation in the marine bacterium *Pseudoalteromonas* sp. 1398 as well. In particular higher [Ca^{2+}] results in reduced amounts of flagella and flagellin production, while it causes an increase in the amounts of at least four other proteins, including a putative phosphatase. Since these increases occurred under both planktonic and biofilm conditions, it appears that calcium has a greater regulatory influence for these extracellular proteins than does biofilm formation.

The source of the bacterial isolate *Pseudoalteromonas* sp. 1398 was sea water sediments. The coexistence of environmental factors (elevated [Ca^{2+}] and surface-associated growth) may serve as complementary signals, and cause similar and/or cumulative changes in the bacterial responses. Cluster analysis of protein profiles observed by 2DGE demonstrated that both surface-associated growth and the presence of Ca^{2+} had a major physiological effect on *Pseudoalteromonas* sp. 1398, and the presence of both factors had cumulative effects on protein profiles. Following surface-associated growth at 0·25 mM $CaCl_2$, *Pseudoalteromonas* sp. had 174 protein differences from planktonically grown cultures, including 46 newly appeared proteins. This response represents a change in approximately 22 % of the total number of intracellular proteins resolved by 2DGE. At 10 mM $CaCl_2$, we observed 302 protein differences, representing approximately 38 % of the resolved proteome. This value included 80 protein spots that were only observed in the biofilm culture. Increased [Ca^{2+}] was positively correlated to an increase in the number of newly appearing protein spots. However, the number of proteins showing reduced amounts did not change in response to $CaCl_2$ addition. When the cluster-analysis comparison was performed independently on proteomes of planktonic cultures, or on the proteomes of surface-associated cultures, we found that Ca^{2+} affects the bacteria differently, with a greater effect seen in the sessile biofilm cultures than in the planktonic cells. The number of proteins showing increased amounts, as well as the number of newly appearing proteins at higher concentrations of calcium, was greater for the biofilm-cultivated cells than for the planktonic bacteria. In addition, there was a positive correlation between the increasing [Ca^{2+}] and number of newly appeared proteins in biofilm cultures, but not planktonic samples. The increase in the number of newly appeared proteins in biofilms compared to planktonic cells could reflect two factors. Firstly, it could be a direct effect of the calcium as a signalling molecule for the biofilm bacteria. Secondly, it could be an indirect effect, due to the increased amount of cell biomass associated with the surface. As the cell density increases, the individual bacteria may experience other environmental variables that could influence gene expression of the surface-associated bacteria. This second scenario, although possible, is less likely in these studies, since the biofilms here were sampled at 7 h, and prior to the development of thick biofilms.

Based on cluster analysis of protein-spot signal intensity, we grouped the proteins of interest into five classes. Fifteen proteins representing different classes were then subjected to sequence analysis by N-terminal peptide sequencing and/or MS/MS analysis. Two proteins, succinyl-CoA synthetase and malate dehydrogenase, were definitively identified as class 1 proteins, which show increased amounts in biofilm culture. Since these proteins are involved in central metabolism, they are not likely to be directly involved in bacterial biofilm formation per se, but rather reflect a change in metabolic activity of the cells as they switch from planktonic to biofilm-associated growth. The proteomics studies performed on *Pseudomonas aeruginosa* and *Escherichia coli* biofilms also showed differential amounts of proteins involved in general metabolic activities when comparing planktonic bacteria to biofilm-associated cells (Perrot *et al.*, 2000; Sauer & Camper, 2001; Tremoulet *et al.*, 2002). Included in those studies were malate dehydrogenase, thiamine phosphate pyrophosphorylase, aldehyde dehydrogenase, sugar and amino acid transporters and amino acid metabolism enzymes. Other class I proteins shown here have tentative identifications, and include a conserved hypothetical protein related to a *Pseudomonas syringae* protein, as well as a putative TonB-dependent receptor protein, found in *I. loihiensis* and in a marine *Pirellula* sp. These proteins represent novel proteins not identified as being expressed during the biofilm formation of other organisms, and therefore may represent proteins that are specific for biofilm formation by *Pseudoalteromonas*. Other proteins showing increased amounts in biofilms include two proteins whose identification was not possible. The lack of sequence identity may have been due to the limited sequence information for marine bacteria, or it may reflect amino acid variability in the region of the protein sequenced here.

Class II proteins are of particular interest, since they reflect proteins with increased amounts during calcium-induced biofilm formation. Therefore, these proteins may play a role in the enhanced biofilm thickness that results from calcium addition. As with the class I proteins, a protein involved in central metabolism was identified, PurM. Tentative identifications were made for three other proteins, which include an outer-membrane protein, OmpW, as well as a putative TonB-system biopolymer transport protein. If the biopolymer transport protein is confirmed, it will be consistent with the increased extracellular polysaccharide observed in biofilms with added Ca^{2+}. The other class II proteins could not be positively identified from this amino acid sequence information. Class III and IV proteins included those that had greater expression in planktonic culture. These proteins either are not essential during biofilm growth, or possibly inhibit growth of the bacteria on a surface. One of the class III proteins was sequenced and identified as a probable peroxiredoxin/glutaredoxin protein involved in electron transport. One class IV protein was identified as a putative intracellular protease. The protease had reduced expression in biofilms, and was also down-regulated by Ca^{2+} addition. The other class IV protein had reduced expression

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in biofilms, but showed increasing amounts with increased [Ca^{2+}], indicating an inverse response of these proteins to changes in [Ca^{2+}], during surface-associated growth.

Many of the proteins shown here to be differentially expressed are novel and have no homologues with high sequence identity. Therefore, genetic studies will be necessary to determine the role of these proteins in biofilm formation. The results here indicate that the environmental factors, surface-associated growth and calcium addition, cause global changes in the protein profiles of the marine bacterium *Pseudoalteromonas* sp. 1398. The combination of the two factors shows combinatory effects on the protein profiles. The results also demonstrate that calcium influences *Pseudoalteromonas* sp. 1398 cells, primarily during biofilm growth.

ACKNOWLEDGEMENTS

We thank Dr Keith Cooksey from for providing the *Pseudoalteromonas* sp. 1398 strain. We thank Dr Daniel Wozniak for providing the flagellin antibodies. We thank Dr L. Eltis for support of the MS/MS analyses. We thank David McCourt for the N-terminal sequence analysis. This work was supported by a grant from the Office of Naval Research: N00014-99-1-0678.

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